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(71) Applicant (for all designated States except US): SUGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US).			
(72) Inventor; and (75) Inventor/Applicant (for US only): CLARY, Douglas [US/US]; 164 Midcrest Way, San Francisco, CA 94131 (US).			
(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).			
(54) Title: STUDY AND TREATMENT OF DISEASES RELATED TO SPECIFIC CELLULAR FUNCTIONS OF RECEPTOR PROTEIN TYROSINE KINASES			
(57) Abstract <p>The invention relates to methods of evaluating the specific function of a receptor protein tyrosine kinase in cells by activating the receptor in a ligand independent fashion. In addition, the invention includes methods of identifying compounds that modulate receptor protein tyrosine kinase function. The invention also relates to a method of preventing or treating an abnormal condition caused by an aberration in the function of the C-RET receptor, and specifically to the treatment and prevention of neuro-degenerative disorders by administering a compound that modulates the function of the C-RET receptor.</p>			

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DESCRIPTIONSTUDY AND TREATMENT OF DISEASES RELATED TO SPECIFIC CELLULAR FUNCTIONS OF RECEPTOR PROTEIN TYROSINE KINASES

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Statement of Related Applications

This application is related to U.S. Patent Application No. 60/043,207, filed April 8, 1997, entitled "Methods of Evaluating Specific Cellular Functions of Receptor Protein Tyrosine Kinases in a Ligand Independent Manner" (Lyon & Lyon Docket No. 222/299) and U.S. Patent Application No. 60/051,715, filed July, 3, 1997, entitled "Methods of Preventing and Treating Neurological Disorders with Compounds that Modulate the Function of the C-RET Receptor Protein Tyrosine Kinase" (Lyon & Lyon Docket No. 223/027).

Field of the Invention

The invention described herein relates generally to methods of determining receptor protein tyrosine kinase function in cells or tissues, and to activating a receptor in a ligand independent manner. The invention specifically relates to the discovery that activation of C-RET is necessary for neuronal cell survival. Thus, the invention also relates to methods of preventing or treating disorders in patients suffering from the effects of neuronal degeneration.

Background of the Invention

The following description of the background of the invention is provided to aid in understanding the invention, but is not admitted to be or describe prior art to the invention.

Cellular signal transduction is a fundamental mechanism whereby extracellular stimuli are relayed to the interior of cells. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of proteins. Phosphorylation of amino acids regulates the activity of mature proteins by altering their structure and function.

Phosphate most often resides on the hydroxyl moiety of serine, threonine, or tyrosine amino acids in proteins. Enzymes that mediate phosphorylation of cellular effectors fall into two classes. While protein phosphatases hydrolyze phosphate moieties from phosphoryl protein substrates, protein kinases transfer a phosphate moiety from adenosine triphosphate to protein substrates. The converse functions of protein kinases and protein phosphatases balance and regulate the flow of signals in signal transduction processes.

Protein kinases are divided further into two groups: receptor and non-receptor type proteins. Receptor protein kinases comprise an extracellular region, a transmembrane region, and an intracellular region. Part of the intracellular regions of receptor protein kinases harbor a catalytic domain.

Receptor protein kinases are also divided into three classes based upon the amino acids they act upon. Some phosphorylate serine or threonine only, some phosphorylate tyrosine only, and some phosphorylate serine, threonine, and tyrosine.

Receptor protein tyrosine kinases (RPTKs) are typically activated in the cell when a ligand binds to the extracellular region of the receptor. A model for ligand mediated activation of RPTKs features the ligand bringing the receptors within close proximity to one another. Some ligands are dimers and thereby bring the receptors that bind them into close proximity with one another. By bringing two RPTKs together, ligands place RPTK intracellular catalytic regions in close proximity

to one another such that they cross-phosphorylate. Cross phosphorylation requires not only the dimerization process but also the occurrence of a conformational change preceding phosphorylation. The necessity of the conformational change preceding phosphorylation is illustrated by the fact that some RPTKs, such as the insulin receptor, are pre-dimerized and inactive before binding their activating ligands.

The presence of phosphate moieties on the RPTK intracellular regions constitutes a cellular signal that causes other signal transduction molecules to bind to the RPTK. In this manner, RPTKs propagate the extracellular signal to the cell nucleus, thereby generating messages encoding proteins that cause cellular affects.

Because RPTKs control a variety of cellular functions, any alteration in the normal function of an RPTK can result in an abnormal condition in an organism. For example, differentiation and survival of neuronal cells is dependent upon the proper function of RPTKs. Specifically, it has been shown that the interaction between activated TRK (Barbacid et al., *Biochimica et Biophysica Acta*, 1072:115-127, 1991) and a signaling component such as SHC is important for promoting neuronal cell differentiation and survival. Transgenic mice containing knockout mutations in genes encoding each of the known TRK receptors or the TRK ligand displayed severe neurological dysfunction and, in all cases, important types of neural tissue were absent (Smeyne et al., 1994, *Nature* 368: 246-249; Klein et al., 1994, *Nature* 268: 249-251, 1994).

Specific neurotrophic factors (generally referred to as ligands herein) have also been shown to promote neuronal survival. In particular, glial-derived neurotrophic factor (GDNF) has been identified as a neuronal survival factor (Jing et al., 1996, *Cell* 85:1113-1124; Trupp et al., 1996, *Nature* 381:785-789; Durbec et al., 1996, *Nature* 381:789-793). GDNF

has been shown to bind to a complex of C-RET and another cell surface protein GDNFR- $\alpha$ , which has no intracellular domain.

A frequent hurdle to designing therapeutics for RPTK-related diseases is identifying the function of a particular receptor and relating its activity to a disease or condition. One difficulty in identifying receptor function is that the ligands that activate the receptors are often unknown. In addition, even if a ligand is known, ligands can sometimes bind more than a single type of receptor and thus cause pleiotropic effects in an organism. Because alterations in the function of one RPTK can lead to a diseased state in an organism, determining the function of individual receptors is important for designing compounds that will prevent or treat these diseases.

Because the ligands that activate RPTK receptors are often unknown, various ligand independent methods of activating RPTK intracellular regions have been tried including using antibodies with specific binding affinity to the extracellular region of one type of receptor (Spaargaren et al., 1991, *J. Biol. Chem.* 266: 1733-1739; Xiong et al., 1992, *Proc. Natl. Acad. Sci USA* 89; Clary et al., 1994, *Mol. Biol. Cell.* 5: 549-563). Because an antibody is bifunctional (it contains two polypeptide binding regions), an antibody can activate RPTKs by bringing them in close proximity with one another and thereby stimulating cross phosphorylation.

RPTKs have also been activated by fusing their intracellular regions to other polypeptides. These fused polypeptide constructs are termed "chimeras" since they are single polypeptides constructed from multiple regions of different proteins (Fazioli et al., 1991, *Mol. Cell. Biol.* 11: 2040-2048; Seedorf et al., 1992, *Mol. Cell. Biol.* 12: 4347-4356). One such RPTK chimera comprises an intracellular region

of a RPTK, fused to an epidermal growth factor (EGF) receptor extracellular region, and thus activatable with the EGF ligand.

RPTK intracellular regions have also been activated in a ligand independent fashion by fusing intracellular polypeptides to them that bind bifunctional organic compounds (Spencer et al., 1993, *Science* 262: 1019-1024).

However, these ligand independent methods of activating RPTK intracellular regions are of restricted application. Simple antibody activation of RPTKs lacks versatility since a unique monoclonal antibody must be produced for each unique RPTK under study. The production of effective monoclonal antibodies for multiple RPTKs is prohibitive as monoclonal antibody production is both time consuming and inefficient.

Furthermore, even though the activation of chimeric constructs of RPTK intracellular regions confers versatility (the intracellular regions of RPTKs can be readily interchanged using recombinant DNA techniques), the ligands and organic compounds used to activate the chimeras can cause changes in cells or tissues other than those caused by the activated chimera. For example, the EGF ligand used to stimulate chimeric constructs containing the EGFR extracellular region can also activate endogenous EGFR expressed on the cell surface, sometimes existing at levels undetectable using currently known methods, and thereby interfere with the signal of the RPTK under study.

In an effort to discover novel treatments for diseases, biomedical researchers have designed, synthesized, and tested molecules that modulate protein kinase function in cells. Some organic molecules have been identified as modulators of RPTK function. For example, bis monocyclic, bicyclic, or heterocyclic aryl compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO 94/14808), 1-cyclopropyl-4-pyridyl-quinolones (U.S. Patent No. 5,330,992), styryl

compounds (U.S. Patent No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660), and benzylphosphonic acid compounds (PCT WO 91/15495) have been identified as compounds which modulate the function of protein kinases.

There remains a great need in the medical field for identifying compounds that modulate the function of RPTKs regulating neuronal survival, in particular. Many patients suffer from diseases caused by neuronal degeneration, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, for example. Although neurotrophic factors have been proposed as therapeutic agents for neuronal diseases, they often cannot reach their target receptors since they rapidly degrade in the blood stream and cannot pass through cell membranes or the blood brain barrier. Thus, identifying effective therapeutics for treating neuronal diseases lies in identifying non-peptide compounds which do not rapidly degrade in the blood stream and which can pass through cell membranes and the blood-brain barrier.

#### Summary of the Invention

The invention relates to methods of evaluating the specific function of a receptor protein tyrosine kinase (RPTK) in cells or tissues by activating a receptor in a ligand-independent fashion. The invention also relates to methods of preventing or treating disorders in patients suffering from the effects of neuronal degradation by administering compounds that modulate the function of C-RET to a patient afflicted with such a disorder. In addition, the invention relates to



pharmaceutical compositions of compounds that modulate the function of C-RET.

Thus, in a first aspect, the invention features a method of evaluating RPTK function in cells. The method comprises the following steps: (a) transfecting a nucleic acid vector into cells, where the nucleic acid vector encodes a chimera comprising an extracellular region and an intracellular region, where the intracellular region is from the receptor protein tyrosine kinase; (b) contacting the cells with an antibody, where the antibody has specific binding affinity to the extracellular region; and (c) monitoring an effect on the cells to evaluate the function of the receptor protein tyrosine kinase.

The term "RPTK" refers to receptor protein tyrosine kinase. The invention includes any RPTK with an extracellular region that binds a ligand, a transmembrane region, and an intracellular region. RPTKs phosphorylate proteins on tyrosine residues. The nucleic acid sequence encoding an RPTK of the invention can be isolated from eukaryotic organisms.

The term "extracellular region" refers to a polypeptide portion of a RPTK of the invention that exists outside the cell membrane. A requirement of the extracellular region of chimeras of the invention is that it has specific binding affinity to another molecule, preferably an antibody of the invention. The extracellular region of the chimeras must not bind to any other protein on the cell surface and not normally be expressed on the surface of the specific cell type used in the invention.

The term "antibody" refers to an antibody (e.g., a monoclonal or polyclonal antibody), or antibody fragment, having specific binding affinity to RPTK's or fragments thereof, preferably the extracellular region of the RPTK's.

By "specific binding affinity" is meant that the antibody binds to target (RPTK) polypeptides with greater affinity than it binds to other polypeptides under specified conditions. Antibodies having specific binding affinity to a RPTK may be used in methods for detecting the presence and/or amount of a protein RPTK in a sample by contacting the sample with the antibody under conditions such that an immunocomplex forms and detecting the presence and/or amount of the antibody conjugated to the RPTK. Diagnostic kits for performing such methods may be constructed to include a first container containing the antibody and a second container having a conjugate of a binding partner of the antibody and a label, such as, for example, a radioisotope. The diagnostic kit may also include notification of an FDA approved use and instructions therefor.

The term "polyclonal" refers to antibodies that are heterogenous populations of antibody molecules derived from the sera of animals immunized with an antigen or an antigen functional derivative thereof, preferably an extracellular region of a RPTK. For the production of polyclonal antibodies, various host animals may be immunized by injection with the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species.

"Monoclonal antibodies" are substantially homogenous populations of antibodies to a particular antigen. They may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. Monoclonal antibodies may be obtained by methods known to those skilled in the art. See, for example, Kohler, et al., Nature 256:495-497 (1975), and U.S. Patent No. 4,376,110.

The term "antibody fragment" refers to a portion of an antibody, often the hypervariable region and portions of the surrounding heavy and light chains, that displays specific binding affinity for a particular molecule. A hypervariable

region is a portion of an antibody that physically binds to the polypeptide target.

The term "ligand" as used herein refers to a polypeptide molecule which binds to the extracellular region of a receptor. Examples of ligands include, but are not limited to, nerve growth factor (NGF), which binds to the extracellular region of the TRK receptor, and GDNF which binds to a complex of C-RET and GDNFR- $\alpha$ .

The term "transmembrane region" as used herein refers to the region of a polypeptide that connects the extracellular region to the intracellular region, preferably in a chimera of the invention. The transmembrane region can be isolated from any transmembrane protein and is preferably from the same polypeptide source as the intracellular region. Transmembrane regions are often rich in hydrophobic amino acids, such as phenylalanine, tyrosine, tryptophan, valine, leucine, and isoleucine, which interact with fatty acid moieties of the membrane lipids and thereby anchor the chimera to the cell membrane.

The term "intracellular region" of an RPTK defines any region existing on the inner side of the cell. An intracellular region of a protein of the invention preferably includes the region of an RPTK with catalytic activity, and no other region of the RPTK.

DNA recombinant techniques known in the art provide the means of connecting an RPTK intracellular region to the extracellular region of another RPTK via a transmembrane region. In addition, the intracellular region of an RPTK can be attached to the extracellular region of a protein other than an RPTK, such as a receptor protein phosphatase.

The term "organism" relates to any living being comprised of at least one cell. An organism can be as simple as one eukaryotic cell or as complex as a mammal.

The term "eukaryote" refers to an organism comprised of cells that contain a nucleus. Eukaryotes are differentiated from "prokaryotes" which do not house their genomic DNA inside a nucleus. Prokaryotes include unicellular organisms such as bacteria, while eukaryotes are represented by, for example, yeast, invertebrates, and vertebrates.

The term "mammal" refers preferably to such organisms as mice, rats, rabbits, guinea pigs, sheep, and goats, more preferably to cats, dogs, monkeys, and apes, and most preferably to humans.

The term "evaluating" describes the process of determining the cellular effect of activating an RPTK intracellular region in cells.

The term "function" refers to the effect of activating the intracellular region of one type of RPTK. The function of the intracellular region of one particular type of RPTK can be manifested in a change in the outward appearance of cells, or in a change in the interactions with proteins that bind to the intracellular region of the RPTK, and the like.

The term "transfecting" defines a number of methods to insert a nucleic acid vector or other nucleic acid molecules into a cellular organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt, an electric field, detergent, or DMSO to render the outer membrane or wall of the cells permeable to nucleic acid molecules of interest or use of various viral transduction strategies.

The term "nucleic acid vector" relates to a single or double stranded circular nucleic acid molecule that can be transfected into cells and replicated within or independently of a cell genome. A circular double stranded nucleic acid molecule can be cut and thereby linearized upon treatment with restriction enzymes. An assortment of nucleic acid vectors,

restriction enzymes, and the knowledge of the nucleotide sequences cut by restriction enzymes are readily available to those skilled in the art. A nucleic acid molecule encoding a chimeric receptor can be inserted into a vector by cutting the vector with restriction enzymes and ligating the two pieces together.

The term "contacting" as used herein refers to mixing a solution comprising one or more test compounds with a liquid medium bathing the cells of the methods. The solution comprising the one or more test compounds may also comprise another component, such as dimethylsulfoxide (DMSO), which facilitates the uptake of the one or more test compounds into the cells of the methods. The solution comprising the test compounds may be added to the medium bathing the cells by utilizing a delivery apparatus, such as a pipet-based device or syringe-based device. "Test compound" includes, but is not limited to, both antibodies and compounds of the invention.

The term "compound" refers to a peptide of less than twenty, preferably less than fifteen, preferably less than ten, or most preferably less than eight amino acids in length. The term "compound" preferably refers to a peptidomimetic, more preferably refers to a non-peptide organic molecule, and most preferably refers to a non-peptide synthetic organic molecule. Examples of compounds are included in the Description of the Invention, herein.

The term "peptidomimetic" refers to an organic molecule with a structure related to a natural peptide, and which can exhibit or block the biological activities of the natural peptide.

The term "monitoring" refers to observing the effect of adding test compounds to the cells. The effect can be manifested in cell phenotype or in the interaction between an RPTK intracellular region and a natural binding partner.

The term "effect" preferably describes a change or absence of a change in cell phenotype. "Effect" can also describe a change or an absence of a change in the catalytic activity of the RPTK. "Effect" can also describe a change or an absence of a change in an interaction between the RPTK and a natural binding partner.

The term "cell phenotype" refers to the outward appearance of a cell or tissue or the function of the cell or tissue. Examples of cell phenotype include, but are not limited to, cell size (reduction or enlargement), cell shape, cell proliferation (increased or decreased numbers of cells), cell differentiation (changes in physiological state), cell survival, apoptosis (cell death), or the utilization of a metabolic nutrient (e.g., glucose uptake). Changes or the absence of changes in cell phenotype are readily measured by techniques known in the art.

The term "cell proliferation" refers to the rate at which a group of cells divides. The number of cells growing in a vessel can be quantified by a person skilled in the art when that person visually counts the number of cells in a defined area using a common light microscope. Alternatively, cell proliferation rates can be quantified by laboratory apparatuses that optically measure the density of cells in an appropriate medium.

Cell differentiation and survival are phenomena simply measured by methods in the art. These methods can involve observing the number of cells or the appearance of cells under a microscope with respect to time (days).

The term "apoptosis" as used herein refers to programmed cell death. It has been shown by example herein that activation of C-RET can prolong cell survival and decrease the probability of cell death.

The term "catalytic activity", in the context of the invention, defines the rate at which an RPTK intracellular region phosphorylates a substrate. Catalytic activity can be measured, for example, by determining the amount of a substrate converted to a phosphorylated product as a function of time. Catalytic activity can be measured by methods of the invention by holding time constant and determining the concentration of a phosphorylated substrate after a fixed period of time. Phosphorylation of a substrate occurs at the active-site of a protein kinase. The active-site is normally a cavity in which the substrate binds to the protein kinase and is phosphorylated.

The term "interaction", in the context of the invention, describes a complex formed between a RPTK and a natural binding partner or compound, preferably between a RPTK intracellular region and a natural binding partner or compound.

The term "complex" refers to an assembly of at least two molecules bound to one another. Signal transduction complexes often contain at least two protein molecules bound to one another. For instance, a protein tyrosine receptor protein kinase, GRB2, SOS, RAF, and RAS assemble to form a signal transduction complex in response to a mitogenic ligand.

The term "natural binding partner" refers to polypeptides that bind to a RPTK. Natural binding partners can play a role in propagating a signal in a protein kinase signal transduction process. A change in the interaction between a RPTK and a natural binding partner can manifest itself as an increased or decreased probability that the interaction forms, or an increased or decreased concentration of the RPTK/natural binding partner complex.

A RPTK natural binding partner can bind to a RPTK intracellular region with high affinity. High affinity represents an equilibrium binding constant on the order of  $10^{-6}$

.M or less. In addition, a natural binding partner can also transiently interact with a RPTK intracellular region and chemically modify it. Protein kinase natural binding partners are chosen from a group consisting of, but not limited to, SRC  
5 homology 2 (SH2) or 3 (SH3) domains, other phosphoryl tyrosine binding (PTB) domains, guanine nucleotide exchange factors, protein phosphatases, and other protein kinases. Methods of determining changes in interactions between protein kinases and their natural binding partners are readily available in the  
10 art.

In preferred embodiments of the invention, the method of evaluating a function of a RPTK involves transfecting nucleic acid vectors that encode chimeras that contain an extracellular region of a RPTK selected from the group consisting of TRK,  
15 EGFR, PDGFR, and RET and intracellular regions of an orphan RPTK. Preferably, the orphan RPTK is selected from the group consisting of C-RET, SEK, MCK-10, AXL, TYRO3, MER, EPH, ECF, EEK, ERK, ELK, EHK1, EHK2, SEK, HEK, HEK2, MYK1, CEK9, MYK2, MDK1, IRR, CCK4, RYK, DDR, TYRO10, ROS, LTK, ALK, ROR1, ROR2,  
20 and TOR. Most preferably, the orphan RPTK is C-RET.

The term "orphan RPTK" as used herein refers to any receptor whose primary amino acid sequence is closely related to the primary amino acid sequence of the RPTK family. It is called an orphan receptor because no ligand has been identified  
25 which directly activates it. In addition, the term "orphan receptor" as used herein refers to an RPTK without a known function. Alternatively, orphan RPTKs may be activated by a ligand known in the art, but the ligand has not yet been shown to directly activate the receptor of interest in the art.

30 The nucleic acid sequences of known RPTKs, and thereby their amino acid sequences, can be readily determined by searching a sequence database with the three or four letter name of the RPTK. Examples of sequence databases are the EMBL



and Genbank databases. In addition, the sequence database can be searched by the database accession number for an RPTK. Furthermore, the databases can be searched using sequences of the RPTKs themselves.

5 Genbank database accession numbers are designated in parenthesis for the sequence of the following RPTK genes: *axl* (M76125); *tyr03* (D17517); *mer* (U08023); *eph* (M18391); *eck* (M59371); *eek* (X59290); *erk* (D31661); *elk* (M59814); *ehk1* (S38024); *ehk2* (S68030); *sek* (S57168); *hek* (M83941); *hek2* (S65702); *myk1* (U06834); *cek9* (Z19060); and *mdk1* (X79082). If  
10 desired, an RPTK gene can be cloned from a cDNA library with nucleic acid probes designed from the nucleic acid sequence provided in the database.

RPTKs can be identified by designations other than those  
15 disclosed herein. For example, the RPTK AXL is also referred to in the art as ARK and UFO. TYRO3 is also referred to as SKY, RSE, TIF, and BRT. MER can be referred to as TYRO12, EYK, and C-RYK. ECK is also referred to as SEK2. ERK is also referred to as HEK5, TYRO5, CEK5, and NUK. ELK can be referred  
20 to as CEK6 and HEK6. EHK1 has been referred to as HEK7, CEK7, and BSK. EHK2 is also referred to as HEK12. SEK can be referred to as HEK8, CEK8, TYRO1, and MPK3. HEK has been referred to as HEK4, CEK4, TYRO4, and MEK4. HEK2 is also referred to as CEK10 and TYRO6. MYK1 is also referred to as  
25 TYRO11, HPTK5, and HTK. CEK9 has been also referred to as HEK9. MDK2 is also referred to as MDK1-T1 or T2, EHK-3, and HEK11.

In addition, other as yet unidentified orphan RPTKs exist that can be identified by methods well-known to those skilled  
30 in the art. RPTKs, like protein kinases in general, share a 250-300 amino acid domain that can be subdivided into 12 distinct subdomains that comprise the common catalytic core structure. These conserved protein motifs can be (and have

been) exploited using PCR-based cloning strategies well-known in the art to identify other RPTKs.

RPTK nucleic acid sequences can also be identified by: analyzing sequenced, but unidentified, DNA or RNA or polypeptide sequences from public databases; by probing DNA libraries (e.g., cDNA and genomic libraries) using degenerate probes to sequences conserved in various families and sub-families of such kinases; or by PCR-based cloning using degenerate primers based on conserved sequences for various families and sub-families. For the probing or PCR-based approaches, once one or more clones are identified having sequences corresponding to the probe or primers, the genomic or cDNA insert in that clone can be at least partially sequenced by routine methods to confirm that the cloned sequence actually corresponds to a RPTK, and to provide a unique sequence identifying the full gene. Possession of such a unique sequence provides the full gene sequence by following routine techniques. Likewise, a probe sequence based on a specific homologous RPTK sequence from a different mammalian species can be used to detect the corresponding human version of that gene.

In preferred embodiments of the method of evaluating a function of a RPTK, the cells of the invention and the extracellular region of the chimera are from different species. Preferably, the cells are mammalian, while the extracellular region of the RPTK is isolated from a chicken. The invention also features methods of evaluating a cellular function of an RPTK, in which the antibodies of the invention have specific binding affinity to a TRK extracellular region isolated from a chicken and where the effect is a change in cell phenotype.

In a second aspect, the invention features a method of identifying one or more compounds that modulate the function of a receptor protein tyrosine kinase in a cell. The method comprises the following steps: (a) transfecting a nucleic acid

vector into a cell, where the nucleic acid vector encodes a chimera comprising an extracellular region and an intracellular region, where the intracellular region is from the receptor protein tyrosine kinase; (b) contacting the cells with one or more compounds; (c) contacting the cells with an antibody, where the antibody has specific binding affinity to the extracellular region and (d) monitoring the effect on the cell to identify compounds that modulates the function of the receptor protein tyrosine kinase.

10       The term "modulates" refers to the ability of a test compound (or "modulator") to alter the function of a RPTK. A modulator preferably activates or inhibits the activity of a RPTK depending on the concentration of the compound exposed to a RPTK. A modulator can enhance or inhibit the catalytic  
15 activity of the RPTK by binding to the RPTK. By binding to the RPTK, the compound may inhibit the catalytic activity of the RPTK by blocking interactions between the RPTK and a substrate that it phosphorylates. A compound may activate the RPTK by bringing RPTKs into close proximity with one another such that  
20 they cross phosphorylate and thereby activate more effectively, or by increasing the probability that a catalysis-dependent conformational change occurs in the RPTK.

In addition, a modulator can inhibit the interaction between a RPTK and a natural binding partner by blocking  
25 interactions between amino acids at the interface of the complex. In addition, a modulator may inhibit the activity of a natural binding partner which acts upon the RPTK. Alternatively, a modulator can enhance the interaction between a RPTK and a natural binding partner by forming additional  
30 favorable interactions between the two molecules at the complex interface. A modulator preferably inhibits the catalytic activity of a protein kinase, more preferably activates or inhibits the catalytic activity of a protein kinase depending

on the concentration of the compound exposed to the protein kinase, or most preferably activates the catalytic activity of a protein kinase.

The term "activates" refers to increasing the cellular function of a RPTK. The RPTK function is preferably the interaction with a natural binding partner or catalytic activity. An activator of C-RET is a cellular factor that is responsible for the cellular response of C-RET after it binds to GDNF. C-RET activators include, but are not limited to GRB2 and SOS. Compounds can enhance the interaction between C-RET and GRB2 or SOS and effectively activate the cellular function of C-RET.

The term "inhibit" refers to decreasing the cellular function of a RPTK. The RPTK function is preferably the interaction with a natural binding partner or catalytic activity. A hypothetical example of a protein that decreases the function of the RPTK, C-RET, is a protein phosphatase that dephosphorylates the activated receptor and thereby decreases C-RET's ability to recruit other proteins necessary for the C-RET activation response, such as GRB2 and SOS.

The term "signal transduction pathway" refers to the molecules that propagate an extracellular signal through the cell membrane to become an intracellular signal. This signal can then stimulate a cellular response. The polypeptide molecules involved in signal transduction processes are typically receptor and non-receptor protein tyrosine kinases, receptor and non-receptor protein phosphatases, SRC homology 2 and 3 domains, phosphotyrosine binding proteins (SRC homology 2 (SH2) and phosphotyrosine binding (PTB and PH) domain containing proteins), proline-rich binding proteins (SH3 domain containing proteins), nucleotide exchange factors, and transcription factors.

The term "substrate" as used herein refers to a molecule phosphorylated by a RPTK. RPTKs phosphorylate substrates on tyrosine amino acids.

In preferred embodiments of methods of identifying compounds that modulate the function of an RPTK, the extracellular region of the chimera is an extracellular region of a RPTK selected from the group consisting of TRK, EGFR, PDGFR, and RET, and the intracellular region is an intracellular region of an orphan RPTK. Preferably the orphan RPTK is selected from the group consisting of C-RET, SEK, MCK-10, AXL, TYRO3, MER, EPH, ECK, EEK, ERK, ELK, EHK1, EHK2, SEK, HEK, HEK2, MYK1, CEK9, MYK2, MDK1, IRR, CCK4, RYK, DDR, TYRO10, ROS, LTK, ALK, ROR1, ROR2, and TOR. Most preferably, the orphan RPTK is C-RET.

In preferred embodiments of the method of identifying compounds that modulate a function of a RPTK, the cells of the invention and the extracellular region of the chimera are from different species. Preferably, the cells are mammalian, while the extracellular region of the RPTK is isolated from a chicken. The invention also features methods of evaluating a cellular function of an RPTK, in which the antibodies of the invention have specific binding affinity to a TRK extracellular region isolated from a chicken and where the effect is a change in cell phenotype. The change in cell phenotype includes, but is not limited to, a change in the catalytic activity of the RPTK intracellular region and a change in an interaction between the intracellular region and a natural binding partner.

In a third aspect, the invention relates to methods of identifying compounds that modulate the function of C-RET RPTK comprising the following steps: (a) transfecting C-RET into cells; (b) contacting the cells with one or more compounds; and (c) monitoring an effect on said cells. The effect includes, but is not limited to, a change or an absence of a change in a

cellular event selected from the group consisting of: c phenotype, which includes, but is not limited to, apoptosis and cell proliferation; catalytic activity of C-RET; and the interaction between C-RET and a natural binding partner.

5 In a fourth aspect, the invention relates to a method of preventing or treating an abnormal condition in a mammal, where the abnormal condition is caused by an aberration in cell survival. The method comprises the step of administering a pharmaceutical composition comprising one or more compounds to  
10 the mammal, where the one or more compounds modulate C-RET.

The term "preventing" refers to decreasing the probability that an organism contracts or develops an abnormal condition.

The term "treating" refers to having a therapeutic effect and at least partially alleviating or abrogating an abnormal  
15 condition in the organism.

The term "therapeutic effect" can refer to the inhibition of cell death causing or contributing to a neuronal disorder. The term "therapeutic effect" can also refer to the inhibition, or activation factors causing or contributing to the abnormal  
20 condition. A therapeutic effect relieves to some extent one or more of the symptoms of the abnormal condition. In reference to the treatment of a neuronal disorders, a therapeutic effect refers to one or more of the following: (a) an increase in the proliferation, growth, and/or differentiation of neuronal  
25 cells; (b) inhibition (i.e., slowing or stopping) of neuronal cell death; (c) inhibition of neuronal degeneration; (d) relieving to some extent one or more of the symptoms associated with the abnormal condition; and (e) enhancing the function of the affected neuronal population (e.g., increase in neurotransmitter synthesis). Compounds demonstrating efficacy against  
30 neuronal disorders can be identified as described herein.

The term "abnormal condition" refers to a function in the cells or tissues of an organism that deviates from their normal

functions in that organism. An abnormal condition can relate to cell proliferation, cell differentiation, or cell survival.

Abnormal cell proliferative conditions include cancers such as fibrotic and mesangial disorders, abnormal angiogenesis and vasculogenesis, wound healing, psoriasis, diabetes mellitus, and inflammation.

Abnormal differentiation conditions include, but are not limited to neurodegenerative disorders, slow wound healing rates, and slow tissue grafting healing rates.

Abnormal cell survival conditions relate to conditions in which programmed cell death (apoptosis) pathways are activated or abrogated. A number of protein kinases are associated with the apoptosis pathways. One RPTK associated with cell survival is C-RET. Aberrations in the function of any one of the protein kinases could lead to cell immortality or premature cell death.

The term "aberration", in conjunction with the function of an RPTK in a signal transduction process, refers to a RPTK that is over- or under-expressed in an organism, mutated such that its catalytic activity is lower or higher than wild-type protein kinase activity, mutated such that it can no longer interact with a natural binding partner, is no longer modified by another protein kinase or protein phosphatase, or no longer interacts with a natural binding partner.

The term "administering" relates to a method of incorporating a compound into cells or tissues of an organism. The abnormal condition can be prevented or treated when the cells or tissues of the organism exist within the organism or outside of the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. For cells harbored within the organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, injection, and aerosol applications. For

cells outside of the organism, multiple techniques exist in the art to administer the compounds, including (but not limited to) cell microinjection techniques, transformation techniques, and carrier techniques.

5       The abnormal condition can also be prevented or treated by administering a compound to a group of cells having an aberration in a signal transduction pathway to an organism. The effect of administering a compound on organism function can then be monitored. The organism is preferably a frog, more  
10       preferably a mouse, rat, rabbit, guinea pig, or goat, and most preferably a monkey or ape.

      The term "pharmaceutical composition" refers to a mixture of the compound with other chemical components, such as diluents or carriers. The pharmaceutical composition  
15       facilitates administering the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds with  
20       organic or inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methane sulfonic acid, ethane sulfonic acid, *p*-toluene sulfonic acid, salicylic acid and the like.

      The term "pharmaceutically acceptable" or "pharmaceutical"  
25       as used herein refers to solutions or components of the pharmaceutical composition that do not prevent the therapeutic compound from exerting a therapeutic effect and do not cause unacceptable adverse side effects. Examples of pharmaceutically acceptable reagents are provided in *The United States Pharmacopeia*, *The National Formulary*, *United States Pharmacopeial Convention, Inc.*, Rockville, MD 1990 and *FDA Inactive Ingredient Guide* 1990, 1996 issued by the Division of Drug Information Resources. Unacceptable side effects vary for  
30



different diseases. Generally, the more severe the disease the more toxic effects which will be tolerated. Unacceptable side effects for different diseases are known in the art.

5 The term "physiologically acceptable" defines a carrier or diluent that does not cause significant irritation to an organism and preferably does not abrogate the biological activity and properties of the compound.

10 The term "carrier" defines a chemical compound that facilitates the incorporation of a compound into cells or tissues. For example dimethyl sulfoxide (DMSO) is a commonly utilized carrier as it facilitates the uptake of many organic compounds into the cells or tissues of an organism.

15 The term "diluent" defines chemical compounds diluted in water that will dissolve the compound of interest as well as stabilize the biologically active form of the compound. Many salts dissolved in buffered solutions are utilized as diluents in the art. One commonly used buffered solution is phosphate buffered saline because it mimics the salt conditions of human blood. Because buffer salts can control the pH of a solution  
20 at low concentrations, a diluent rarely modifies the biological activity of a compound.

In preferred embodiments of methods of preventing or treating an abnormal condition in a mammal, the RPTK is C-RET. Preferably, the method at least partially reduces the symptoms  
25 associated with the abnormal condition, which is preferably caused by an aberration in cell survival.

The term "aberration in cell survival" as used herein refers to a condition in which cell survival is enhanced or decreased with respect to cell survival parameters in normal  
30 cells. Cell survival can be shortened in such disorders as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. Cell survival can be increased in cancer related conditions.

Alzheimer's disease manifests itself as presenile dementia. The disease is characterized by confusion, memory failure, disorientation, restlessness, speech disturbances, and hallucination in mammals (Medical, Nursing, and Allied Health Dictionary, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

Parkinson's disease is a slowly progressive, degenerative, neurologic disorder characterized by resting tremor, loss of postural reflexes, and muscle rigidity and weakness (Medical, Nursing, and Allied Health Dictionary, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

Amyotrophic lateral sclerosis is a degenerative disease of the motor neurons characterized by weakness and atrophy of the muscles of the hands, forearms, and legs, spreading to involve most of the body and face (Medical, Nursing, and Allied Health Dictionary, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

In preferred embodiments of methods of preventing or treating an abnormal condition in a mammal, the abnormal condition is a neurodegenerative disorder, preferably selected from the group consisting of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis. Preferred methods at least partially prevent or reduce a symptom associated with the abnormal condition.

The term "neurodegenerative disorder" as used herein refers to an abnormality in a mammal in which neuronal integrity is threatened. Neuronal integrity can be threatened when neuronal cells display decreased survival or when the neurons can no longer propagate a signal.

In another preferred embodiment, the invention relates to the method of treating or preventing an abnormal condition in the mammal, where the mammal is identified as having said abnormal condition. Mammals can be identified as having, for

example, a neurological disorder such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis by monitoring the symptoms manifested in the mammals afflicted with these disorders. These symptoms are well documented in publications known to those skilled in the art.

In other preferred embodiments of methods of treating or preventing an abnormal condition in a mammal, the pharmaceutical composition is comprised of one or more compounds that are non-peptide molecules and one or more physiologically acceptable solvents, diluents, or carriers. Preferably these compounds act intracellularly and can traverse the blood-brain barrier. Preferably the compounds activate the catalytic activity of the RPTK, inactivates an inhibitor of the RPTK, or activates an activator of the RPTK. Most preferably, the RPTK is C-RET.

The term "non-peptide molecule" refers to a compound that is not a polymer of amino acids. A non-peptide molecule preferably does not contain chemical moieties that hydrolyze in physiological conditions, i.e. a peptidomimetic.

The term "solvent" as used herein refers to a chemical compound that facilitates the solubilization of compounds of the invention. Examples of solvents include, but are not limited to, pharmaceutically acceptable alcohols, such as ethanol and benzyl alcohol; polyoxyhydrocarbyl compounds, such as poly(ethylene glycol); pharmaceutically acceptable surfactants such as CREMOPHOOR EL; polyglycolized lipids, such as GELUCIRE and LABRASOL; and pharmaceutically acceptable oils, such as miglyol 812.

The term "pharmaceutically acceptable alcohol" as used herein refers to alcohols which are liquids at about room temperature (approximately 20°C). These include propylene glycol, ethanol, 2-(2-ethoxyethoxy)ethanol (TRANSCUTOL®, Gattefosse, Westwood, NJ 07675), benzyl alcohol, and glycerol.

The term "polyoxyhydrocarbyl compound" as used herein refers to a water soluble carbohydrate such as glucose, sucrose, maltotriose, and the like; water soluble carbohydrate derivatives such as gluconic acid and mannitol, and oligosaccharides; and water soluble polymers such as polyvinylpyrrolidone, poly(vinyl alcohol), and in particular, polyethers such as other polyoxyalkylenes including poly(ethylene glycol) or other water soluble mixed oxyalkylene polymers and the polymeric form of ethylene glycol. Although polyoxyhydrocarbyl compounds preferably contain more than one carbon, oxygen, and hydrogen atom, some molecules such as poly(ethylene imine) are also included.

A particularly preferred class of solubilizing polyoxyhydrocarbyl moieties comprises poly(ethylene glycol) (PEG) and PEG derivatives, such as PEG monomethyl ether. Other suitable PEG derivatives include PEG-silicon derived ethers. Many of these polymers are commercially available in a variety of molecular weights. Others may be conveniently prepared from commercially available materials, such as by coupling of amino-PEG moiety to a haloalkyl silyl or silane moiety.

Suitable PEGs may vary in molecular weight from about 200 g/mol to about 20,000 g/mol or more, more preferably 200 g/mol to 5,000 g/mol, even more preferably 250 g/mol to 1,000 g/mol, and most preferably 250 g/mol to 500 g/mol. The choice of a particular molecular weight may depend on the particular indolinone-based compound chosen and its molecular weight and degree of hydrophobicity, as well as the particular application for which the formulation is to be used.

The term "pharmaceutically acceptable surfactant" as used herein refers to a compound that can solubilize compounds of the invention into aqueous solutions. Preferably for parenteral formulations, the surfactant is a non-ionic surfactant. Examples of pharmaceutically acceptable

surfactants include POLYSORBATE 80® and other polyoxyethylene sorbitan fatty acid esters, glyceryl monooleate, polyvinyl alcohol, ethylene oxide copolymers such as PLURONIC™ (a polyether) and TETRONIC™ (BASF), polyol moieties, and sorbitan esters. Preferably ethoxylated castor oils, such as CREMOPHOR EL®, are used for the formulation of indolinone-based compounds.

The term "ethoxylated castor oil" as used herein refers to castor oil that is modified with at least one oxygen containing moiety. In particular the term refers to castor oil comprising at least one ethoxyl moiety.

Further, the term "pharmaceutically acceptable surfactant" as used herein in reference to oral formulations, includes pharmaceutically acceptable non-ionic surfactants (for example polyoxyethylenepolypropylene glycol, such as POLOXAMER® 68 (BASF Corp.) or a mono fatty acid ester of polyoxyethylene (20) sorbitan monooleate (TWEEN® 80), polyoxyethylene (20) sorbitan monostearate (TWEEN® 60), polyoxyethylene (20) sorbitan monopalmitate (TWEEN® 40), polyoxyethylene (20) sorbitan monolaurate (TWEEN® 20) and the like); polyoxyethylene castor oil derivatives (for example, polyoxyethyleneglycerol-triricinoleate or polyoxyl 35 castor oil (CREMOPHOR® EL, BASF Corp.), polyoxyethyleneglycerol oxystearate (CREMOPHOR® RH 40 (polyethyleneglycol 40 hydrogenated castor oil) or CREMOPHOR® RH 60 (polyethyleneglycol 60 hydrogenated castor oil), BASF Corp.) and the like); or a pharmaceutically acceptable anionic surfactant.

The term "polyglycolized lipids" as used herein refers to mixtures of monoglycerides, diglycerides, or triglycerides and polyethyleneglycol monoesters and diesters formed by the partial alcoholysis of vegetable oil using PEG of 200 g/mol to 2,000 g/mol or by the esterification of fatty acids using PEG 200 g/mol to 2,000 g/mol and glycerols. Preferably these

include GELUCIRE® 35/10, GELUCIRE® 44/14, GELUCIRE® 46, GELUCIRE® 50/13, GELUCIRE® 53/10, and LABRASOL®.

The term "pharmaceutically acceptable oils" as used herein refers to oils such as mineral oil or vegetable oil (including safflower oil, peanut oil, and olive oil), fractionated coconut oil, propylene glycol monolaurate, mixed triglycerides with caprylic acid and capric acid, and the like. Preferred embodiments of the invention feature mineral oil, vegetable oil, fractionated coconut oil, mixed triglycerides with caprylic acid, and capric acid. A highly preferred embodiment of the invention features Miglyol 812 (available from Huls America, USA).

The term "acts intracellularly" as used herein refers to a compound that can modulate the function of a RPTK by binding to the intracellular region of the RPTK. In addition, the compound can bind to a natural binding partner of the RPTK intracellular region.

The "blood-brain" barrier is an anatomic feature of the brain that is thought to comprise walls of capillaries in the central nervous system combined with surrounding glial membranes (Medical, Nursing, and Allied Health Dictionary, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

A final aspect of the invention features pharmaceutical composition comprising one or more compounds and one or more physiologically acceptable solvents or diluents or carriers. The one or more compounds of the pharmaceutical composition can modulate a RPTK cellular function, preferably activate or inhibit a RPTK cellular function, and most preferably modulates C-RET.

The summary of the invention described above is not limiting and other features and advantages of the invention

will be apparent from the following detailed description of the invention, and from the claims.

#### Detailed Description of the Invention

5       The invention relates to methods of evaluating the specific function of a receptor protein tyrosine kinase (RPTK) in cells or tissues by activating a receptor in a ligand-independent fashion. The invention also relates to methods of preventing or treating disorders in patients suffering from the  
10       effects of neuronal degradation by administering pharmaceutical compositions comprised of compounds that modulate the function of C-RET to a patient afflicted with such a disorder.

      The ligand-independent methods of evaluating the specific function of a RPTK overcome at least two problems associated  
15       with studying RPTKs and identifying compounds that modulate RPTK function. These problems are (1) lack of knowledge about and availability of the ligand that activates a RPTK, and (2) ligands may activate more than one type of RPTK or activate undetectable levels of endogenous receptor, and thereby cause  
20       effects in a test cell that are not truly associated with the receptor under study.

      The invention features antibody-induced activation of chimeric RPTKs constructed from polypeptides of at least two different proteins. The chimeras contain the extracellular and  
25       transmembrane region from one type of RPTK and an intracellular region from another type of RPTK. This chimeric approach confers both specificity and versatility towards evaluating RPTK cellular function.

      The methods of the invention incorporate a novel cross  
30       species feature with respect to the chimeric constructs and cells utilized in these methods. The cross species feature ensures that the only receptor appreciably activated in response to an antibody of the invention is the chimeric

construct expressed on the surface of cells. This cross species feature represents an improvement over the existing techniques which can activate multiple receptors on cell surfaces.

5       Therefore, the methods set forth herein provide for test compound-induced activation of specific chimeric receptors expressed on the surface of cells under study. As an example, the extracellular region of the chimeric construct may originate from chickens while the cells in which the chimeric  
10       construct is expressed originate from rats. Examples set forth herein demonstrate that antibodies raised specifically to the chicken extracellular region of the chimeric construct do not cross-react with other proteins expressed on rat cell surfaces.

      RPTKs are essential regulatory molecules controlling a  
15       variety of cellular functions. For this reason, any alteration in the function of a RPTK can result in an abnormal condition in an organism. Just two of the many functions controlled by RPTKs are cell proliferation and cell survival. Aberrations in cell survival can lead to neurological disorders.

20       The methods of preventing or treating disorders in patients suffering from the effects of neuronal degradation are based in part on the discovery that activation of C-RET is necessary for neuronal cell survival. C-RET has been implicated in neuronal survival by methods of the invention  
25       that activate the receptor in a ligand independent fashion. These methods utilize antibodies specific for the TRK extracellular region of a TRK extracellular region/C-RET intracellular region chimeric receptor. The antibodies specifically activate the chimeric receptor by bringing the C-  
30       RET intracellular regions in close proximity such that they cross phosphorylate and thereby activate. These methods were utilized to discover the function of C-RET before the ligand



that activates this receptor, GDNF, was known to those skilled in the art.

Identifying effective therapeutics for treating neuronal diseases lies in identifying non-peptide compounds that  
5 activate C-RET. Non-peptide compounds are preferred since they are often stable in a patient's blood stream. In addition, non-peptide compounds can be selected such that they can pass through cell membranes and the blood-brain barrier. Although  
10 many of the non-peptide molecules discovered to modulate the function of RPTKs only inhibit their targets, activating compounds of RPTKs are being discovered.

#### I. Cell Proliferation and Signal Transduction

Alterations in the function of an RPTK that normally  
15 regulates cell proliferation can lead to enhanced or decreased cell proliferative conditions evident in certain diseases. Aberrant cell proliferative conditions include cancers, fibrotic disorders, mesangial disorders, abnormal angiogenesis and vasculogenesis, poor wound healing, psoriasis, restenosis,  
20 and inflammation.

Fibrotic disorders relate to the abnormal formation of the cellular extracellular matrix. An example of a fibrotic disorder is hepatic cirrhosis. Hepatic cirrhosis is characterized by an increased concentration of extracellular  
25 matrix constituents resulting in the formation of a hepatic scar. Hepatic cirrhosis can cause diseases such as cirrhosis of the liver.

Mesangial cell proliferative disorders occur due to the abnormal proliferation of mesangial cells. Mesangial  
30 proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombotic micro angiopathy syndromes, transplant rejection, and glomerulopathies.

Angiogenic and vasculogenic disorders result from excessive proliferation of blood vessels. Blood vessel proliferation is necessary in a variety of normal physiological processes such as embryonic development, corpus luteum formation, wound healing and organ regeneration. However, blood vessel proliferation is also essential in cancer tumor development. Other examples of blood vessel proliferative disorders include arthritis, where new capillary blood vessels invade the joint and destroy cartilage, and ocular diseases (such as diabetic retinopathy), where new capillaries in the retina invade the vitreous, bleed and cause blindness. Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated in adverse regulation of RPTKs.

15

## II. Neurological Disorders and Signal Transduction

Abnormalities in signal transduction pathways can lead to various diseases through both underactivity and over activity of the RPTKs involved in these pathways. Examples of disorders which are characterized by underactivity of a RPTK in a signal transduction pathway include various neurodegenerative diseases such as myasthenia gravis, amyotrophic lateral sclerosis, cervical spondylosis, and Alzheimer's disease. A neurological disease possibly characterized by over activity of an RPTK in a signal transduction pathway is neurofibromatosis. See, generally, The Merck Manual of Diagnosis and Therapy, 16th Edition, 1992.

The development and maintenance of cellular communication networks within the central and peripheral nervous system is regulated by neurotrophic factors, which through activation of specific cell surface receptors generate differentiation and survival signals in neuronal cell types. Binding of the neurotrophic factor to its receptor initiates a cellular signal

transduction cascade involving diverse cytoplasmic components which eventually results in a specific nuclear response. Specific cellular responses in nerve cells can include, for example, neurite outgrowth, acquisition of Na<sup>+</sup>-based action potential and cell survival in serum-free medium.

The complex processes involved in cell survival are mediated by diverse and divergent signal transduction pathways. The multiple phosphorylated tyrosines found in activated RPTKs serve as binding sites for different signaling components, which in turn modulate the transduction of a signal along a particular pathway.

In the case of the NGF receptor TRK, it has been shown that specific phosphorylated tyrosines within the cytoplasmic portion of the receptor can bind to the signaling components phospholipase C-gamma (PLC-gamma), SHC and the non-catalytic subunit of phosphatidylinositol-3'-kinase, p85 (Obermeier, A., *EMBO Journal* 12:933-941, 1993 and Obermeier, A., *J. Biol. Chem.* 268:22963-22966, 1993). These proteins in turn each stimulate different and distinct further downstream signaling components until instructions are finally transmitted to the cell nucleus. For example, SHC has been shown to bind to the GRB2/SOS complex, which in turn allows the activation of RAS. Activation of PLC-gamma, on the other hand, leads to the generation of phosphatidylinositol metabolites, such as inositol 1,4,5-triphosphate, which cause the release of calcium ions from intracellular compartments and the generation of diacylglycerol, the natural activator of PKC.

Until recently, it was not yet understood which RPTKs were responsible for regulating cell survival. Methods described in the invention demonstrate that C-RET regulates cell survival in neurons.

### III. Method of Determining the Function of C-RET

The following method which was used to demonstrate that C-RET regulates neuronal survival, can also be used to determine the function of other RPTK receptors. This method specifically  
5 activates RPTKs in cells in a ligand-independent fashion. The RPTKs are activated with antibodies specific to the extracellular region of a chimeric receptor. The chimeric receptor can contain an extracellular region and an intracellular region from two different RPTKs. Specifically, a  
10 chimera can contain the extracellular region of the TRK receptor and an intracellular region of C-RET. Activation of such a chimeric construct resulted in the prolonged survival of various neuronal cells. Examples of such studies are provided herein.

15 A chimera, as used in the context of the invention, defines a polypeptide constructed from polypeptides of at least two different proteins. The chimeric polypeptides are encoded by nucleic acid molecules harbored by a nucleic acid vector transfected into cells. Hence, a chimera can contain the  
20 extracellular and transmembrane region from one type of RPTK and the intracellular region from another type of RPTK.

The chimeric feature of the method provides versatility as the intracellular region of virtually any known RPTK can be incorporated readily into a chimeric construct using DNA  
25 recombinant techniques existing in the art. The chimeras comprise a polypeptide extracellular region, a transmembrane region, and an intracellular region of a RPTK. The chimeric polypeptides are encoded by nucleic acid molecules harbored by a nucleic acid vector transfected into cells or tissues. The  
30 extracellular region of the chimera is a polypeptide region that exists outside of the cell and specifically binds to an antibody of the method. The intracellular region of the

chimera exists inside the cell and comprises any part of a RPTK intracellular region.

The chimeric feature of the method also confers specificity as chimeras can be activated in a specific manner by an antibody having specific binding affinity to the polypeptide extracellular region. An antibody can specifically bind to a single type of extracellular region of a chimera on cell surfaces. As antibodies are "Y-shaped" molecules that can bind two polypeptide molecules, antibodies can bring the chimeras in close proximity to one another in the cell. Once the intracellular regions of the chimera are in close proximity to one another, they can cross phosphorylate one another and become activated. The effect of a compound on the cellular function of a RPTK can then be measured by monitoring changes in cell phenotype, catalytic activity of the RPTK, or interactions of the RPTK with natural binding partners. Hence, antibody-induced activation of chimeras specifically activates RPTK intracellular regions without causing other cellular effects.

To achieve specific activation of chimeric receptor constructs, the method features a cross species chimera/cell system. In this system, the extracellular region of the chimeric receptor is selected from an organism distantly related to the organism from which the cells expressing the chimeric receptor are derived. This cross species strategy ensures that endogenous receptors expressed on the cell surface are not activated by the antibodies raised against the extracellular region of the chimeric construct. For example, the antibodies of the invention have specific binding affinity for the TRK extracellular region from chicken. The antibodies having specific binding affinity for the chicken TRK extracellular region will consequently not cross react with the endogenous TRK receptor of rat or human cells when the chimeric

constructs are expressed in these cells. Thus, the antibodies of the invention prevent cross reactivity with endogenous receptors expressed on the surface of mammalian cells since the antibodies have specific binding affinity to a distinct TRK receptor of a different type of organism.

#### IV. Function of C-RET in Cells

Using the method described above, it was determined that C-RET regulates cell survival. Signal transduction molecules that form a complex with C-RET as a result of these phosphoryl moieties, such as GRB2, SOS, RAS, and RAF, propagate a signal in the cell that promotes neural survival. Thus, compounds that promote the interactions of these stimulatory molecules of C-RET would enhance the activity of C-RET. Alternatively, protein phosphatases can remove the phosphoryl moieties placed on the intracellular region of C-RET in response to GDNF, and thus inhibit the signaling capability of C-RET. Thus compound that inhibit phosphatases of C-RET will enhance the signaling capacity of C-RET.

C-RET is implicated in the development and survival of enteric, synaptic, and sensory neurons and neurons of the renal system upon stimulation by GDNF (Jing et al., 1996, Cell 85:1113-1124; Trupp et al., 1996, Nature 381:785-789; Durbec et al., 1996, Nature 381:789-793). Lack of function mutations in C-RET can lead to Hirschsprung's disease, for example, which manifests itself as a decrease in intestinal tract innervation in mammals. Thus, compounds that activate C-RET are potential therapeutic agents for the treatment of neurodegenerative disorders, including, but not limited to, Hirschsprung's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. Compounds that inhibit C-RET function are also possible anti-cancer agents as over-

expression of RET in cells is implicated in cancers, such as cancer of the thyroid.

Modulation of C-RET activity may also be useful in treating cancers of the nerve tissue, such as neuroblastoma, even if an abnormality is not found the signaling pathway.

#### V. Therapeutics for Neurological Disorders

The development of effective therapeutics for treating and preventing neurological disorders lies in identifying non-peptide compounds which do not rapidly degrade in the blood stream and which can pass through cell membranes and the blood-brain barrier. Several companies are attempting to treat neurodegenerative diseases (such as amyotrophic lateral sclerosis) by administering exogenous recombinant neurotrophic factors (such as BDNF) to mammals, in the hope that the factor will stimulate new growth and differentiation of nerve cells. Although administration of neurotrophic factors may stimulate the growth and differentiation of nerve cells grown on plates, the neurotrophic factors will likely be rapidly degraded in the blood stream by proteases since they are composed of amino acids. In addition, the neurotrophic factors cannot pass through cell membranes or the blood brain barrier. In order to treat Parkinson's disease or Alzheimer's disease, these therapeutics need to reach the brain in order to be efficacious.

For these reasons, non-peptide compounds are likely candidates as therapeutics for neurological disorders. These compounds must be selected for their ability to activate C-RET in order to treat or prevent neurological diseases. The methods set forth herein can select non-peptide molecules as activators of C-RET. In addition, select compounds are probable activators of C-RET. These compounds are set forth in WO 96/40113 and its

related priority documents. Examples of indolinone compounds are also set forth herein by example.

#### VI. Pharmaceutical Formulations and Routes of Administration

5       The compounds described herein can be administered to a human patient per se, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical  
10       Sciences," Mack Publishing Co., Easton, PA, latest edition.

##### a) Routes Of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct  
15       intraventricular, intravenous, intraperitoneal, intranasal, c intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the  
20       compound directly into a solid tumor, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with  
25       tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

##### b) Composition/Formulation

The pharmaceutical compositions of the present invention  
30       may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.



Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl

pyrrolidone, agar, or alginic acid or a salt thereof such sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which  
5 may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different  
10 combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the  
15 active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or  
20 liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

25 For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-  
30 rotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler

or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation

(for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably, without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semi-

permeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the PTK modulating compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms.

#### c) Effective Dosage

Pharmaceutical compositions suitable for use in the present invention include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTK activity). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data; e.g., the concentration necessary to achieve 50-90% inhibition of the kinase using the

assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

5 Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

10 In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

#### d) Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of a tumor, inhibition of angiogenesis, treatment of fibrosis, diabetes, and the like.

30

#### VII. Other Embodiments

Method for evaluation of disorders, methods for monitoring changes in cells, methods of identifying compounds, methods of

isolating compounds which interact with a RPTK, compositions  
compounds that interact with a RPTK, and derivatives of  
complexes are disclosed in detail with respect to the protein  
PYK-2 in PCT publication WO 96/18738. Those skilled in the art  
5 will appreciate that such descriptions are applicable to the  
present invention and can be easily adapted to it. Those  
skilled in the art will also appreciate that any modifications  
made to a complex can be manifested in a modification to any of  
the molecules in that complex. Thus, the invention includes  
10 any modifications to nucleic acid molecules, polypeptides,  
antibodies, or compounds in a complex.

In addition, the WO 96/18738 provides disclosure describ-  
ing the recombinant DNA techniques pertaining to the present  
invention, nucleic acid vectors, the nucleic acid elements of  
15 these vectors, the types of cells that can harbor these  
vectors, methods of delivering these vectors to cells or  
tissues, methods of producing and purifying antibodies, metho  
of constructing hybridomas that produce these antibodies,  
methods of detecting signaling molecule complexes, methods of  
20 detecting interactions with natural binding partners,  
antibodies to complexes, disruption of RPTK protein complexes,  
purification and production of complexes, transgenic animals  
containing nucleic acid vectors encoding a RPTK, antisense and  
ribozyme approaches, and gene therapy techniques. Those  
25 skilled in the art will readily appreciate that such  
descriptions are applicable to the present invention and can be  
easily adapted to it.

Other methods associated with the invention are described  
in the examples disclosed herein.

30

#### Examples

The examples below are not limiting and are merely  
representative of various aspects and features of the present



invention. The examples below demonstrate methods of identifying compounds that modulate the function of C-RET. In addition, the examples below demonstrate compounds that activate RPTKs. Furthermore, the examples below demonstrate methods used to identify the role of C-RET in cell survival. The examples also demonstrate methods of specifically activating RPTKs in a ligand-independent manner. The examples demonstrate the specificity as well as the versatility of the methods.

10 EXAMPLE 1: Methods of Identifying Compounds That Modulate the Function of C-RET

An enzyme linked immunosorbant assay (ELISA) was conducted to measure the catalytic activity of C-RET and more specifically, the inhibition or activation of compounds on the catalytic activity of C-RET. The following assay was conducted to measure catalytic activity of C-RET in PC12CTAret cells.

The PC12CTAret cells were created by techniques well known to those skilled in the art. For example, the C-RET gene was subcloned from a nucleic acid vector described in the following examples into a nucleic acid vector suitable for stably transfecting PC12 cells. These nucleic vectors are commonly and commercially available for such purposes. The methods of cloning the genes of interest into these nucleic acid vectors and the methods of transfecting these nucleic acid constructs into cells are also well known to those skilled in the art (Sambrook, Fritsch, Maniatis, *Molecular Cloning, A Laboratory Manual 2nd Ed.*, Cold Spring Harbor Laboratory Press, 1989).

The materials and protocol for the C-RET ELISA assay are as follows:

30

Materials:

- a. Falcon 10-cm tissue culture dishes (Falcon Cat. #3003);
- b. Corning 96-well ELISA plates (Corning Cat. # 25805-96);

- c. Falcon 96-well tissue culture plates (Falcon Cat. #3072);
- d. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);
- e. PC12CTAret (PC12 cells expressing a chimeric form of C-RET: extracellular domain of chicken TRKA and transmembrane and intracellular domains of human C-RET, with an HA epitope tag fused to carboxyl terminus);
- 5 f. Collagen I, bovine (Collaborative Biomedical, Cat. # 40231);
- 10 g. NGF (nerve growth factor). 25  $\mu$ g/mL stock (prepared in: 100 mM NaCl, 50 mM sodium acetate, pH 5.3) (Austral Biologicals Cat. # GF-022);
- h. dimethyl sulfoxide (100%) (Sigma Catalog No. D-8418);
- i. PBS,  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free (Gibco Cat. # 14190-029);
- 15 j. Culture medium for PC12CTAret:  
RPMI (Gibco Cat. # 11875-093)
  - + 10% horse serum, (Gibco Cat # 16050-023),
  - + 5% fetal bovine serum (Gibco Cat # 16000-028),
  - + 1% Penicillin-Streptomycin (Gibco Cat 15140015),
  - 20 + 400  $\mu$ g/mL G418 (Geneticin<sup>TM</sup>; Gibco Cat # 11811-031);
- k. TBST Buffer (50 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween-20);
- l. HNTG Buffer (20 mM HEPES buffer pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 5 mM EDTA, 1 mM sodium orthovanadate, 5 mM NaPyrophosphate);
- 25 m. Anti-HA monoclonal antibody, clone 12CA5 (Boehringer Mannheim Cat. # 1666606);
- n. Biotinylated anti-phosphotyrosine monoclonal antibody, 4G10-Biotin. (Upstate Biotechnology Cat. # 16-103);
- 30 o. Europium-labeled Streptavidin (Wallac Cat. # 1244-360);
- p. Assay buffer (Wallac Cat. # 1244-106); and
- q. Enhancement solution (Wallac Cat. # 1244-105);

Protocol

1. PC12CTAret was subcloned in a PC12 cell line that expresses a chimeric HA-tagged c-ret receptor under the CMV promoter (vector pcDNA3, Invitrogen). The cells were passed  
5 when they were 60-70% confluent (about  $1 \times 10^7$  cells/plate), usually 1:6 once every four days. Cells can be removed from the plates by pipetting medium over them several times.
2. Pre-coat the 96- well cell plates with collagen I. Dilute the collagen stock to 10  $\mu\text{g/mL}$  with PBS  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -  
10 free and add 100  $\mu\text{L}$  per well. Let stand at least 15', and shake out.
3. Harvest the PC12CTAret cells in 5 mL of growth medium (without G418) by pipetting up and down several times. After counting, adjust the cell suspension to have  $5 \times 10^5$  cells/mL so  
15 that  $5 \times 10^4$  cells are seeded by adding 100  $\mu\text{L}$  per well. The plates are assayed the next day.
4. Coat Corning 96-well ELISA plates with 0.5  $\mu\text{g/well}$  12CA5 in PBS buffer (100  $\mu\text{L}$  at 5 mg/mL) 2 hours at room temperature with agitation.
- 20 5. Dilute drugs to 10 X concentration in RPMI or RPMI containing 10% DMSO such that each working dilution is 10% DMSO final concentration.
6. NGF is diluted to 40 ng/mL in complete PC12CTAret medium.
- 25 7. Add NGF and drugs to the cells. 13  $\mu\text{L}$  of diluted NGF is added per well, followed by 13  $\mu\text{L}$  of 10 X concentrated drug. Shake gently and put back in  $\text{CO}_2$  incubator for 90 minutes.
8. For wells to be used as positive receptor phosphorylation controls add NGF to 50 ng/mL final  
30 concentration for the last 15 minutes.
9. After the coating period with capture antibody, remove unbound antibody from the ELISA plates by inverting; rinse twice with TBST.

50

10. After the 90' incubation with drug, remove medium from cell plates by inverting, pat on paper towels to absorb excess liquid and add 100  $\mu$ L HNTG\* buffer. Shake vigorously for 5 min. to complete lysis.
- 5 11. Transfer lysate to coated ELISA and incubate at room temperature for 1 h with agitation.
12. Remove lysate by inverting plates, wash twice with TBST and pat on paper towels to absorb excess liquid.
13. Add 100  $\mu$ L detection antibody 4G10-Biot. at 75 ng/mL  
10 in TBST. Incubate for 60 minutes at room temperature with agitation.
14. Remove detection antibody and wash twice with TBST as in step 12.
15. Add 100  $\mu$ L Eu-Streptavidin at 50 ng/mL in Assay  
15 Buffer. Incubate for 30 minutes at room temperature with agitation.
16. Remove Eu-Streptavidin and wash four times with TBST as in step 12.
17. Add 100  $\mu$ L Enhancement Solution, shake for 5 minutes  
20 at room temperature.
18. Determine assay results by analyzing the plate fluorescence on Wallac DELFIA 1234 fluorometer.

25 EXAMPLE 2: Methods of Identifying Compounds That Modulate the  
Function of RPTKs

An enzyme linked immunosorbant assay (ELISA) was conducted to measure the catalytic activity of the FLK-1 receptor and more specifically, the inhibition or activation of indolinone compounds of the catalytic activity of the FLK-1 receptor. The  
30 following assay was conducted to measure catalytic activity of the FLK-1 receptor in FLK-1/NIH3T3 cells.

One skilled in the art can adapt such a procedure to other receptor tyrosine protein kinases to identify compounds that modulate the function of the receptor.

This type of assay can be developed for nearly any RPTK using well known techniques utilized by persons of ordinary skill in the art. The FLK-1/NIH3T3 cell lines were also created by techniques well known to those skilled in the art. The NIH3T3 cells were stably transfected with gene constructs containing the flk-1 gene. The nucleic acid vectors used for this type of procedure are commonly and commercially available to those skilled in the art. The methods of cloning the genes of interest into these nucleic acid vectors and the methods of transfecting these nucleic acid constructs into cells are also well known to those skilled in the art (Sambrook, Fritsch, Maniatis, Molecular Cloning, A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, 1989).

The materials and protocol for the FLK-1 ELISA assay are as follows:

Materials

- a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);
- b. Cappel goat anti-rabbit IgG (catalog no. 55641);
- c. PBS (Gibco Catalog No. 450-1300EB);
- d. TBSW Buffer (50 mM Tris (pH 7.2), 150 mM NaCl and 0.1% Tween-20);
- e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4 °C);
- f. HNTG buffer (20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 10% glycerol);
- g. EDTA (0.5 M (pH 7.0) as a 100X stock);
- h. Sodium ortho vanadate (0.5 M as a 100X stock);
- i. Sodium pyro phosphate (0.2 M as a 100X stock);

- j. NUNC 96 well V bottom polypropylene plates (Appl. Scientific Catalog No. AS-72092);
- k. NIH3T3 C7#3 Cells (FLK-1 expressing cells);
- l. DMEM with 1X high glucose L Glutamine (catalog No. 11965-050);
- 5 m. FBS, Gibco (catalog no. 16000-028);
- n. L-glutamine, Gibco (catalog no. 25030-016);
- o. VEGF, PeproTech, Inc. (catalog no. 100-20) (kept as 1  $\mu\text{g}/100 \mu\text{L}$  stock in Milli-Q  $\text{dH}_2\text{O}$  and stored at  $-20^\circ\text{C}$ ;
- 10 p. Affinity purified anti-FLK-1 antiserum;
- q. Monoclonal antibody specific for phosphotyrosine (see, Fendley, et al., 1990, Cancer Research 50:1550-1558);
- r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011);
- 15 s. 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) solution (100 mM citric acid (anhydrous), mM  $\text{Na}_2\text{HPO}_4$  (pH 4.0), 0.5 mg/mL ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at  $4^\circ\text{C}$  until ready for use;
- 20 t.  $\text{H}_2\text{O}_2$  (30% solution) (Fisher catalog no. H325);
- u. ABTS/ $\text{H}_2\text{O}_2$  (15 mL ABTS solution, 2  $\mu\text{L}$   $\text{H}_2\text{O}_2$ ) prepared 5 minutes before use and left at room temperature;
- v. 0.2 M HCl stock in  $\text{H}_2\text{O}$ ;
- 25 w. dimethyl sulfoxide (100%) (Sigma Catalog No. D-8418); and
- y. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049).

Protocol:

- 30 1. Coat Corning 96-well ELISA plates with 1.0  $\mu\text{g}$  per well Cappel Anti-rabbit IgG antibody in 0.1 M  $\text{Na}_2\text{CO}_3$  pH 9.6. Bring final volume to 150  $\mu\text{L}$  per well. Coat plates overnight

at 4 °C. Plates can be kept up to two weeks when stored at 4 °C.

2. Grow cells in Growth media (DMEM, supplemental with 2.0 mM L-Glutamine, 10% FBS) in suitable culture dishes until  
5 confluent at 37 °C, 5% CO<sub>2</sub>.

3. Harvest cells by trypsinization and seed in Corning 25850 polystyrene 96-well round bottom cell plates, 25,000 cells/well in 200 µL of growth media.

4. Grow cells at least one day at 37 °C, 5% CO<sub>2</sub>.

10 5. Wash cells with D-PBS 1X.

6. Add 200 µL/well of starvation media (DMEM, 2.0 mM L-Glutamine, 0.1% FBS). Incubate overnight at 37 °C, 5% CO<sub>2</sub>.

7. Dilute Compounds/Extracts 1:20 in polypropylene 96 well plates using starvation media. Dilute dimethyl sulfoxide  
15 1:20 for use in control wells.

8. Remove starvation media from 96 well cell culture plates and add 162 µL of fresh starvation media to each well.

9. Add 18 µL of 1:20 diluted Compound/Extract dilution (from step 7) to each well plus the 1:20 dimethyl sulfoxide  
20 dilution to the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethyl sulfoxide is 0.5%. Incubate the plate at 37 °C, 5% CO<sub>2</sub> for two hours.

10. Remove unbound antibody from ELISA plates by inverting plate to remove liquid. Wash 3 times with TBSW +  
25 0.5% ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.

11. Block plates with TBSW + 0.5% Ethanolamine, pH 7.0, 150 µL per well. Incubate plate thirty minutes while shaking on a micro titer plate shaker.

30 12. Wash plate 3 times as described in step 10.

13. Add 0.5 µg/well affinity purified anti-FLU-1 polyclonal rabbit antiserum. Bring final volume to 150 µL/well

with TBSW + 0.5% ethanolamine pH 7.0. Incubate plate for thirty minutes while shaking.

14. Add 180  $\mu$ L starvation medium to the cells and stimulate cells with 20  $\mu$ L/well 10.0 mM sodium ortho vanadate and 500 ng/mL VEGF (resulting in a final concentration of 1.0 mM sodium ortho vanadate and 50 ng/mL VEGF per well) for eight minutes at 37 °C, 5% CO<sub>2</sub>. Negative control wells receive only starvation medium.

15. After eight minutes, media should be removed from the cells and washed one time with 200  $\mu$ L/well PBS.

16. Lyse cells in 150  $\mu$ L/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium ortho vanadate, sodium pyro phosphate and EDTA.

17. Wash ELISA plate three times as described in step 10.

18. Transfer cell lysates from the cell plate to ELISA plate and incubate while shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.

20. Incubate ELISA plate with 0.02  $\mu$ g/well UB40 in TBSW + 0.5% ethanolamine. Bring final volume to 150  $\mu$ L/well. Incubate while shaking for 30 minutes.

21. Wash plate three times as described in step 10.

22. Incubate ELISA plate with 1:10,000 diluted EIA grade goat anti-mouse IgG conjugated horseradish peroxidase in TBSW + 0.5% ethanolamine, pH 7.0. Bring final volume to 150  $\mu$ L/well. Incubate while shaking for thirty minutes.

23. Wash plate as described in step 10.

24. Add 100  $\mu$ L of ABTS/H<sub>2</sub>O<sub>2</sub> solution to well. Incubate ten minutes while shaking.

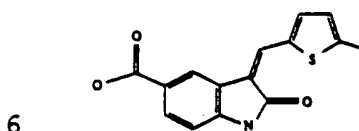
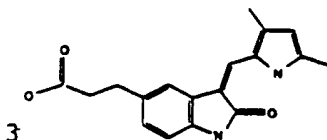
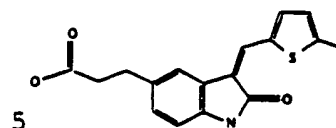
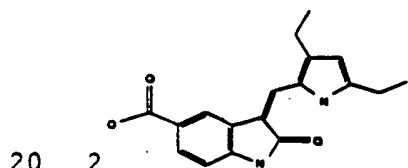
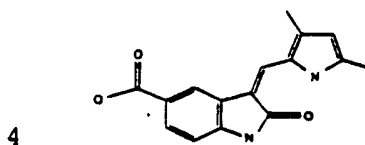
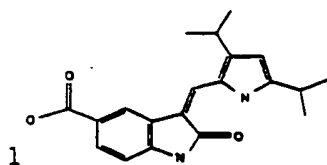
25. Add 100  $\mu$ L of 0.2 M HCl for 0.1 M HCl final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader.



### EXAMPLE 3 Potential Therapeutics for Neurological Disorders

Selected compounds are known to inhibit particular receptor protein tyrosine kinases with high affinity and specificity. For example, see International Patent Publication Number WO 96/22976 for descriptions of indolinone compounds. Most of the indolinone compounds specified inhibit RPTKs with high affinity, but many of them do not enhance the catalytic activity of these protein kinases.

However, the following indolinone compounds activated selected RPTKs. EC<sub>200</sub> values were measured for the following indolinone compounds in the FLK-1 ELISA. EC<sub>200</sub> values are the concentration of the indolinone compound that yielded a two-hundred percent increase in catalytic activity in the FLK-1 ELISA.



The EC<sub>200</sub> values for indolinones 1 through 6 are as follows:

TABLE I

Indolinone	EC <sub>200</sub> ( $\mu$ M)
1	10
2	2.8
3	32
4	2.8
5	>100
6	46

5 EXAMPLE 4: Specific Activation of RPTKs by Antibodies

The following example demonstrates that the intracellular region of a RPTK can be activated when fused to a TRK extracellular region isolated from chicken. This chimeric construct was activated by an antibody that had specific binding  
10 affinity for the TRK extracellular region from chicken organisms. In particular, the chimeric constructs were activated by polyclonal antibody preparation raised against the chicken TRKA receptor. The antibody specifically activated the chimeric construct in cells, since the cells did not express  
15 any other proteins harboring regions homologous to the TRK extracellular region from chicken. Thus, the antibodies distinguished between endogenous TRKA receptor expressed on the surface of the rat cells, and the chimeric construct containing the chicken TRKA extracellular region, also expressed on the  
20 surface of the cells.

The antibody preparation used in the experiments, called CTA, was a rabbit antibody raised against the entire extracellular domain of chicken TRKA expressed in COS cells. The antibody was specific for chicken TRKA and did not cross-react with the rat receptor, nor did it elicit biological activities in rat cells. It did, however, strongly recognize the chicken TRKA receptor by immunoblot or immunoprecipitation and acted as a ligand which activated the chicken receptor. A useful control for these experiments was to activate the endogenous rat TRKA receptor without influencing the introduced chimeric receptor. This specific activation was achieved through the use of the RTA IgG preparation, which recognized the rat receptor but not the chicken receptor.

An assumption made in the experiments was that the chimeric receptor activated by the CTA antibody did not in turn activate the endogenous receptor. The assumption was tested by expressing both rat and chicken TRKA receptors in the same cell and activating them with either neurotrophic growth factor (NGF), which binds both receptors, or with the CTA antibody, which binds only the chicken receptor. While the chicken receptor became phosphorylated by both treatments, activation of the rat receptor was detected only after NGF addition. Thus, the activated chicken receptor did not in turn activate the rat receptor. The effects of the CTA antibody preparation were therefore not attributable to activation of endogenous TRKA receptors.

The sympathetic and sensory neurons used in the experiments were cultured in a defined medium (Awrot and Patterson, 1979, *Methods Enzymol.* 53: 574-584). Sympathetic neurons were isolated from superior cervical ganglia dissected from E20 - E21 rat fetuses, while dorsal root ganglion sensory neurons were obtained from E16 - E18 rats. The ganglia were treated with 0.25% trypsin for 10 minutes, washed, and

triturerated to obtain a single cell suspension. Sensory neurons were pre-plated for 1 hour on tissue culture plastic to deplete adherent cells. Sensory and sympathetic neurons were infected with adenoviruses directing expression of RPTKs that comprise the chicken extracellular domain for two hours on collagen I-coated tissue culture plastic in the presence of NGF. The cells were then washed and allowed to recover for two to four additional hours in the presence of NGF. After the recovery period, the cells were washed extensively to remove the growth factor, and plated onto polylysine-laminin coated chamber slides with 15  $\mu\text{g/mL}$  RTA or CTA IgG, or no addition. After an additional two days (sensory) or three days (sympathetic), the cultures were stained with calcein AM (1  $\mu\text{g/mL}$ ) for 45 minutes, mounted and examined by immunofluorescence. Generally, five disperse fields representing 7% of the well were photographed and the number of surviving neurons quantified.

The conditions for immunoprecipitation and immunoblots used in the experiments were described previously (Clary et al., 1994, *Mol. Biol. Cell* 5: 549-563) with modifications as follows. Immunoblots were performed in Tris-buffered saline containing 0.1% Triton X-100, and filter blocking and antibody incubations were performed in the same buffer with 1% BSA. In other experiments, anti-phosphotyrosine detection was performed with biotinylated-4G10 monoclonal antibody followed by a peroxidase avidin-biotin complex. Other antibodies were detected with peroxidase coupled anti-mouse IgG, anti-rabbit IgG, or protein A as indicated. All peroxidase conjugates were detected using a chemiluminescence protocol (Pierce Chemical Co. Rockford, IL).

EXAMPLE 5: Construction of Adenoviral Vectors Expressing  
Receptor Constructs

In order to test the biochemical effect of receptor tyrosine kinases in chimeric receptor constructs, a method for efficient gene transfer was developed which worked with multiple cell lines as well as primary neuronal cultures. A recombinant adenoviral system was developed, as adenovirus exhibits a wide host range, and can infect and direct gene expression in post-mitotic neurons.

Recombinant adenoviruses were generated by in vivo ligation. The transfer vector (pAdRSVOES-) contained 5' adenovirus packaging sequences, the Rous Sarcoma Virus long terminal repeat promoter, a polylinker ending in the restriction site BstBI, the SV40 poly A region, 3' adenoviral sequences (which could be used for recombination), and sequences for propagation in *E. coli*. The viral DNA used for generation of recombinant viruses was derived from a virus which expressed the  $\beta$ -galactosidase gene driven by the RSV promoter, and which contained a BstBI site between the  $\beta$ -galactosidase gene and the poly A region. This vector allowed screening for recombinant plaques based on the presence or absence of  $\beta$ -galactosidase activity, similar to a system described by Schaack and colleagues (Schaack et al., 1995, *J. Virol.* 69: 3920-3923). This virus lacks the E3 region.

The cells utilized for the generation of recombinant adenovirus were HEK293 cells. Early passage HEK293 cells (Graham et al., 1977, *J. Gen. Virol* 36: 59-72) were maintained in Dulbecco's modified Eagles medium + 10% calf serum. Cells infected with recombinant adenovirus were detected as plaques in the cell monolayer. Typically, 5  $\mu$ g of transfer vector plasmid DNA cleaved by digestion with BstBI were co-precipitated with 2  $\mu$ g of viral DNA also cleaved with BstBI. HEK293 monolayers were transfected with the DNA and cultured

from five to seven days to allow plaques to appear. T. monolayers were then stained with 25  $\mu$ g/mL X-GAL (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) for several hours to identify non-recombinant (stained) plaques. Putative recombinant plaques were screened for expression of the transgene by infection of HEK293 cultures followed by immunohistochemistry. Viruses which were positive for transgene protein expression were picked and subjected to several rounds of plaque purification prior to amplification and purification on cesium chloride gradients. Banded viruses were diluted five-fold with dilution buffer (Curiel et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8850-8854) and stored at -80 °C. Approximate titers of the virus preparations were determined immunohistochemically on HEK293 cultures. Although recombinant viruses were generated through recombination into the viral genome or through co-transfection of cleaved DNAs, higher recovery of recombinants was achieved through the cleaved DNA method. The putative recombinant viruses were screened for expression of the kinase transgene by immuno-histochemistry.

Immuno-histochemistry techniques were performed on cultures of sympathetic neurons infected with the RSV KP or RSV c-ret viruses and fixed and stained as described (Lefcort et al., 1996, J. Neurosci. 16: 3704-3713). The primary antibodies were the CTA IgG, and mouse anti-tyrosine hydroxylase. The primaries were detected with Cy3-anti rabbit IgG and fluorescein-anti mouse IgG.

#### EXAMPLE 6: Expression and Activation of C-RET, SEK, and MCK-10 Chimeras

Once the adenoviral expression vectors were transfected into the neuronal cells, an initial survey of RPTK expression was performed for these cells. Several uncharacterized kinases were expressed in these cell types. For example, the RPTK SFK

was expressed in embryonic rat motor neurons, while MCK-10 (DDR) and C-RET were expressed in both motor and sympathetic neurons.

DNA constructs were generated, which encoded the transmembrane and cytoplasmic domains from human MCK-10, C-RET, mouse SEK, and rat TRKA fused to the chicken TRKA extracellular domain. These chimeric receptor constructs were then inserted into the adenovirus transfer vector, and recombinant viruses were generated and purified. Rat C6 glioma cells were infected with the recombinant viruses, and the resulting chimeric receptors were analyzed via immunoblot analysis.

The chimeric ret protein appeared to be approximately 155 kDa as expected. The chimeric SEK protein was approximately 150 kDa, which is very similar to the chimeric and non-chimeric TRKA constructs, which have a molecular weight of approximately 140 kDa, and consistent with a fully processed, mature receptor. The MCK-10 chimeric protein appeared in three bands, of molecular weights of approximately 160, 120, and 105 kDa. The two lower molecular weight forms were quite abundant and probably represent immature forms of this chimera.

Once it was determined that the chimeric receptors were expressed in the cells, their activity was determined by monitoring the degree of autophosphorylation after stimulation by ligand (e.g. the TRKA CTA antibody).

The ability to activate the chimeras was evaluated by incubating the infected C6 monolayers with 50 ng/mL NGF for five minutes. The receptors were immunoprecipitated with the CTA antibody, immunoblotted, and probed either with the anti-phosphotyrosine antibody 4G10 or with the CTA antibody preparation. NGF-dependent activation of the C-RET and SEK chimeras was detected by the anti-phosphotyrosine antibody. The chimeric C-RET receptor showed substantial activation in the absence of ligand addition. Phosphotyrosine was not

detected for the MCK-10 chimeric protein, either before after incubation with NGF.

The PC12 and C6 glioma cells used in the experiments were cultured as follows. PC12 cultures (Greene et al., 1987, Methods Enzymol. 147: 207-216) were maintained in RPMI medium containing 10% horse serum and 5% fetal calf serum. For differentiation experiments the medium was changed to RPMI containing IX N2 supplement and 0.1% BSA, and the cells were grown on a collagen I substrate. For PC12 cell survival, the cells were grown in RPMI containing 0.1% BSA. All cultures also contained IX penicillin/streptomycin. For adenoviral infections, PC12 cells were incubated overnight with recombinant viruses at a multiplicity of infection (MOI) between 1 and 10. The cells were then washed and re-plated either into differentiation or survival conditions, with either no addition or addition of 50 ng/mL (NGF) or 20 µg/mL CTA IgG for two days.

For differentiation experiments, the cell cultures were fixed with 2% paraformaldehyde and the percentage of cell-bearing processes longer than 1 cell diameter was determined. For survival, the cultures were incubated with 0.05% MTT for 1.5 hours to stain living cells, and the relative number of cells surviving in each condition was determined.

C6 glioma cells were grown in Ham's F10 medium containing 10% fetal calf serum; for receptor activation experiments the medium was changed to 0.5% fetal calf serum 12 hours prior to stimulation.

#### EXAMPLE 7: The C-RET Chimeric Receptor Promotes Differentiation and Survival of PC12 Cells

The biological activities of the chimeric receptor constructs were tested after it was determined that these constructs were expressed and active when stimulated with the CTA antibody, as shown in Example 6. As a first test of the



biological activities of these chimeric constructs, their effects on the differentiation and serum-free survival of the pheochromocytoma cell line PC12 were tested.

Lysates from PC12 cultures prepared two days after  
5 infection with viruses were probed for receptor expression with the CTA antibody. Strong expression of the full length and chimeric TRKA and MCK-10 receptors was observed. Lower levels of the C-RET and SEK receptors were expressed. As expected, the CTA antibody did not react with uninfected cultures or  
10 cultures infected with the  $\beta$ -galactosidase virus.

To examine the effects of these receptors on cell differentiation, the PC12 cells were first treated with virus overnight, and then re-plated under conditions which promote cellular differentiation. The cultures were incubated either  
15 in the presence of CTA IgG, to activate the introduced receptors, or NGF, to activate both endogenous and exogenous pools. The CTA antibody preparation could promote extensive outgrowth of the PC12 cells infected with the c-ret chimeric virus as well as with the full length and chimeric trkA  
20 viruses. The antibody had no obvious effect on RSV sek or RSV mck-10.

The percentage of cells undergoing differentiation was quantified in each condition. RSV c-ret was able to promote differentiation of PC12 cells to a level comparable to that  
25 seen with either trkA construct. RSV c-ret virus showed a much higher activity in the absence of ligand than the trkA viruses did, most likely due to its auto-activation. No detectable activity of the sek or mck-10 constructs was observed.

The effects of the viruses on PC12 cell death was also  
30 tested. PC12 cells undergo programmed cell death in the absence of serum, and can be rescued by NGF. PC12 cells infected with the recombinant adenoviruses were tested for the ability of CTA antibody to rescue these cells from serum

deprivation. The cells were infected as described above, a. plated with and without CTA Antibody in serum free medium. MTT was used to detect surviving cells in the cultures two days later. The strongest effect on cell survival was observed for the two TRKA receptor constructs. The C-RET chimeric receptor could prevent cell death as well, but its effect reached a plateau at a level below that of the *trkA* viruses. The RSV *sek* and RSV *mck-10* viruses had marginal if any effect on the rescue of cells from death.

Hence, the C-RET cytoplasmic domain had biological activities comparable to those of TRKA in promoting PC12 cell differentiation and PC12 cell survival, while these activities were not detected after transfection with the *sek* or *mck-10* receptor chimeras.

15

#### EXAMPLE 5: Activation of the C-RET Chimera Promotes Survival of Embryonic Sympathetic Neurons

C-RET was activated in a ligand-independent manner and tested for its ability to prevent apoptosis in embryonic sympathetic neurons. Sympathetic neurons extend processes and maintain viability and electrical excitability for weeks when cultured in the presence of NGF. In the absence of NGF, the neurons lose their processes, shrivel and undergo a classical program of apoptosis within hours. Activation of the TRKA receptor can prevent apoptosis in sympathetic neurons without the addition of NGF.

The expression of chimeric receptor constructs was examined in rat sympathetic neurons by infecting the neurons, culturing them in the presence of RTA antibody, and lysing them 48 hours later. The RTA antibody was used to maintain their viability in the event that expression of a TRKA chimera would inhibit the function of the endogenous rat TRKA when NGF was used as a ligand. The lysates were immunoblotted and probed

with the CTA antibody. Strong expression of RSV KP (TRKA chimera), RSV c-ret, and RSV sek was observed. RSV mck-10 showed somewhat weaker expression, especially of the 160 kDa isoform.

5       The neurons were transfected and plated in order to examine the effects of the chimeric receptors on neuronal cell survival. The infected neurons were cultured in the presence of RTA antibody to activate their endogenous TRKA receptor, CTA antibody to activate the introduced receptor, or no addition to  
10 examine the background of cell survival. After three days, the cultures were stained with the vital dye calcein AM, and the number of surviving neurons in each condition was determined.

Uninfected sympathetic neurons responded well to the RTA antibody preparation, while the CTA antibody was ineffective in  
15 promoting cell survival. RTA could also sustain the survival of neurons infected with the RSV KP, RSV c-ret, RSV sek, or RSV mck-10 viruses. Moreover, infection of the neurons with RSV KP (the chimeric trkA virus) resulted in the ability of the neurons to respond to CTA, and the level of survival measured  
20 in the presence of CTA reached levels equal to the survival promoted by RTA antibody. This indicated that survival of primary neurons could be promoted by introduction and activation of RPTKs.

Increased survival of sympathetic neurons was also  
25 observed when they were infected with the RSV c-ret virus and cultured with the activating CTA antibody. The level of survival reached approximately 50% of the maximal level attained with the RSV KP virus. In addition, a substantial increase in survival in the absence of the activating antibody  
30 was measured. About 30% of the neurons survived compared to RSV KP plus the activating antibody. This result was not unexpected, given the relatively high level of autoactivation detected with this chimeric receptor. Even though there was

significant survival in the RSV KP wells in the absence of C antibody, it was not as striking as with RSV c-ret. Finally, neither the RSV sek nor the RSV mck-10 virus could increase the survival of the neurons in the presence of CTA antibody, in contrast to the survival seen with RSV KP and RSV c-ret.

The cells surviving through infection with the RSV c-ret virus were tested for the proper phenotype of a superior cervical ganglion neuron. Neurons were cultured with or without NGF for two days following infection with either the RSV c-ret or the RSV KP virus. The cultures were then subjected to immunofluorescence. In all cases neurons which were strongly expressing the chimeric receptors exhibited a healthy phenotype. When the cultures were supplemented with NGF, the cells expressing the transgene comprised a subset of surviving neurons, while in the absence of NGF, all large healthy neurons were found to be expressing the chimeric receptor. In addition, the neurons expressed comparable level of tyrosine hydroxylase as compared to RSV KP controls. In cultures sustained by infection with the RSV c-ret virus only, the levels of tyrosine hydroxylase appeared to be highest in the cells expressing the highest levels of chimeric receptor, indicating that C-RET may be acting like the TRKA receptor in up regulating tyrosine hydroxylase in activity.

#### EXAMPLE 6 Prevention of Sensory Neuron Programmed Cell Death by the C-RET Intracellular Region

c-ret constructs were transfected into DRG embryonic rat sensory neurons to determine whether the RPTK could promote survival in these cells as well as the sympathetic neurons studied in example 5.

All three of the receptors were robustly expressed in the DRG neuronal cell cultures. After plating the cells, both the TRKA and C-RET chimeric receptors could promote a significant

level of neuronal survival, while the *sek* receptor construct was inactive. The level of survival promoted by C-RET in the presence of the CTA antibody was about 50% of that promoted by RSV KP, and again there was significant survival in the absence  
5 of the antibody (31% of that measured with RSV KP plus CTA antibody). Therefore, the survival activity of the C-RET receptor intracellular region extends to at least two major cell types, sympathetic and sensory neurons, in the peripheral nervous system.

10

EXAMPLE 7: TRKA and C-RET Chimeric Receptors Promote MAPK  
Phosphorylation in Primary Neurons

The molecular mechanisms behind cell survival were explored once it was observed that activation of the C-RET  
15 chimera stimulated cell survival, as demonstrated in Examples 5 and 6. In particular, the relationship between the C-RET intracellular region and the proteins that it potentially phosphorylated were explored.

Analysis of downstream signal transduction effectors of  
20 the TRKA and C-RET receptors showed significant similarities in certain effector systems. For example, GRB2 has been shown to bind one isoform of the C-RET receptor at its cytoplasmic tail; this interaction is predicted to activate the RAS/RAF/MAPK pathway. TRKA activates this pathway through interaction of the  
25 SHC adaptor protein with a phosphotyrosine located in the juxta membrane region. Experiments were performed which determined that survival-promoting receptors could activate the MAPK pathway in sympathetic neurons.

Neurons were infected with virus constructs carrying  
30 either active or inactive *trkA* receptor genes or the chimeric *c-ret* receptor gene. The neurons were cultured in the presence of the RTA antibody for two days, and then starved for 3 hours. Half of the wells were stimulated for ten minutes with the CTA

antibody, the cultures were lysed, and the resulting lysate immunoblotted. The immunoblots were probed with antibodies recognizing MAPK p42 and p44 (ERK1 and ERK2) or probed with antibodies recognizing only the phosphorylated form of MAPK.

5 CTA stimulation of the RSV KP and RSV c-ret receptors efficiently and equivalently promoted MAPK phosphorylation. In addition, significant phosphorylation of MAPK with RSV c-ret was observed in the absence of stimulation, consistent with the observation that this construct was autoactivated. Thus, the  
10 correlation between the roles of TRKA and C-RET in promoting primary neuronal survival and activating the MAPK pathway suggest that the MAPK pathway is a component of the apoptosis mechanism.

One skilled in the art would readily appreciate that the  
15 present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments  
20 are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that  
25 varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the  
30 art to which the invention pertains.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed

herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as  
5 terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various  
10 modifications are possible within the scope of the invention claimed.

In particular, although some formulations described herein have been identified by the excipients added to the formulations, the invention is meant to also cover the final formulation formed by the combination of these excipients.  
15 Specifically, the invention includes formulations in which one to all of the added excipients undergo a reaction during formulation and are no longer present in the final formulation, or are present in modified forms.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the  
20 art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims  
25 for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.

Claims

1. A method of evaluating a function of a receptor protein tyrosine kinase comprising the following steps:
  - (a) transfecting a nucleic acid vector into cells,  
5 wherein said vector encodes a chimera comprising an extracellular region and an intracellular region, wherein said intracellular region is from said receptor protein tyrosine kinase;
  - (b) contacting said cells with an antibody, wherein  
10 said antibody has specific binding affinity to said extracellular region; and
  - (c) monitoring an effect on said cells.
2. The method of claim 1, wherein said extracellular  
15 region consists of an extracellular region of a receptor protein tyrosine kinase selected from the group consisting of TRK, EGFR, PDGFR, and RET.
3. The method of claim 1, wherein said intracellular  
20 region consists of an intracellular region of an orphan receptor protein tyrosine kinase.
4. The method of claim 3, wherein said orphan receptor protein tyrosine kinase is selected from the group consisting  
25 of C-RET, SEK, MCK-10, AXL, TYRO3, MER, EPH, ECK, EEK, ERK, ELK, EHK1, EHK2, SEK, HEK, HEK2, MYK1, CEK9, MYK2, MDK1, IRR, CCK4, RYK, DDR, TYRO10, ROS, LTK, ALK, ROR1, ROR2, and TOR.
5. The method of claim 4, wherein said orphan receptor  
30 protein tyrosine kinase is C-RET.
6. The method of claim 1, wherein said cells and said extracellular region are from different species.



7. The method of claim 6, wherein said cell is mammalian.

5 8. The method of claim 6, wherein said extracellular region is isolated from a chicken.

9. The method of claim 1, wherein said antibody has specific binding affinity to a TRK extracellular region  
10 isolated from a chicken.

10. The method of claim 1, wherein said effect is a change or an absence of a change in cell phenotype.

15 11. A method of identifying compounds that modulate the function of a receptor protein tyrosine kinase in cells, wherein said method comprises the following steps:

(a) transfecting a nucleic acid vector into said cells, wherein said vector encodes a chimera comprising an  
20 extracellular region and an intracellular region, wherein said intracellular region is from said receptor protein tyrosine kinase;

(b) contacting said cells with one or more compounds;

25 (c) contacting said cells with an antibody, wherein said antibody has specific binding affinity to said extracellular region; and

(d) monitoring an effect on said cells.

30 12. The method of claim 11, wherein said extracellular region consists of an extracellular region of a receptor protein tyrosine kinase selected from the group consisting of TRK, EGFR, PDGFR, and RET.

13. The method of claim 11, wherein said intracellular region consists of an intracellular region of an orphan receptor protein tyrosine kinase.

5

14. The method of claim 13, wherein said orphan receptor protein tyrosine kinase is selected from the group consisting of C-RET, SEK, MCK-10, AXL, TYRO3, MER, EPH, ECK, EEK, ERK, ELK, EHK1, EHK2, SEK, HEK, HEK2, MYK1, CEK9, MYK2, MDK1, IRR, CCK4, RYK, DDR, TYRO10, ROS, LTK, ALK, ROR1, ROR2, and TOR.

10

15. The method of claim 14, wherein said orphan receptor protein tyrosine kinase is C-RET.

15

16. The method of claim 11, wherein said cell and said extracellular region are from different species.

17. The method of claim 16, wherein said cells are mammalian.

20

18. The method of claim 16, wherein said extracellular region is isolated from a chicken.

19. The method of claim 11, wherein said antibody has specific binding affinity to a TRK extracellular region isolated from a chicken.

25

20. The method of claim 11, wherein said effect is a change or an absence of a change in cell phenotype.

30

21. The method of claim 11, wherein said effect is a change or an absence of a change in the catalytic activity of said intracellular region.

22. The method of claim 11, wherein said effect is a change or an absence of a change in an interaction between said intracellular region and a natural binding partner.

5

23. A method of identifying compounds that modulate the function of C-RET receptor protein tyrosine kinase comprising the following steps:

- (a) expressing said C-RET in cells;
- 10 (b) contacting said cells with one or more compounds; and
- (c) monitoring an effect on said cells.

24. The method of claim 23, wherein said effect is a  
15 change or an absence of a change in cell phenotype.

25. The method of claim 23, wherein said effect is a change or an absence of a change in catalytic activity of said C-RET receptor.

20

26. The method of claim 23, wherein said effect is a change or an absence of a change in the interaction between said C-RET receptor and a natural binding partner.

25 27. A method of preventing or treating an abnormal condition in a mammal, wherein said abnormal condition is caused by an aberration in cell survival, said method comprising the step of administering a pharmaceutical composition comprising one or more compounds to said mammal, wherein  
30 said compound is a modulator of a receptor protein tyrosine kinase.

28. The method of claim 27, wherein said receptor prote tyrosine kinase is C-RET.

29. The method of claim 27, wherein said abnormal  
5 condition is a neurodegenerative disorder.

30. The method of claim 29, wherein said neurodegenerative disorder is selected from the group consisting of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral  
10 sclerosis.

31. The method of claim 27, wherein said method at least partially prevents or reduces a symptom associated with said abnormal condition.

15

32. The method of claim 27, wherein said compound is a non-peptide molecule.

33. The method of claim 27, wherein said one or more  
20 compounds act intracellularly.

34. The method of claim 27, wherein said one or more compounds can traverse the blood-brain barrier.

25 35. The method of claim 27, wherein said pharmaceutical composition further comprises one or more physiologically acceptable solvents.

36. A pharmaceutical composition comprising one or more  
30 compounds that modulate the function of the C-RET receptor and one or more physiologically acceptable solvents.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 98/06842

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/566 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M SANTORO, W T WONG, P AROCA, E SANTOS, B MATOSKOVA, M GRIECO, A FUSCO, P P DI FIORE: "An Epidermal Growth Factor Receptor/ret Chimera Generates Mitogenic and Transforming Signals: Evidence for a ret-Specific Signaling Pathway" MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 1, January 1994, pages 663-675, XP002071816	1-7, 10-17, 20-22
Y	see the whole document	4,5, 11-17, 20-22
Y	WO 96 22976 A (PHARMACIA SPA ;BUZZETTI FRANCO (IT); BRASCA MARIA GABRIELLA (IT);) 1 August 1996 cited in the application see the whole document	11-17, 20-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel: (+31-70) 340-2040, Tx: 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hart-Davis, J

# INTERNATIONAL SEARCH REPORT

In International Application No

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C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
Y	M TRUPP, E ARENAS, M FANZILBER, A-S NILSSON, B-A SIEBER, M GRIGORIOU, C KILKENNY, E SALAZAR-GRUESO, V PACHNIS, U ARUMAE ET AL.: "Functional receptor for GDNF encoded by the c-ret proto-oncogene" NATURE, vol. 381, 27 June 1996, pages 785-789, XP002071817 cited in the application see the whole document	4.5, 14, 15, 23-36
X	P MCCLOSKEY, J PIERCE, R A KOSKI, B VARNUM, E T LIU: "Activation of the Axl Receptor Tyrosine Kinase " CELL GROWTH AND DIFFERENTIATION, vol. 5, no. 10, October 1994, XP002071818 see the whole document	1-4, 6, 7, 10
Y	---	5, 11-17, 20-22
P, X	J BRAUNGER, L SCHLEITHOFF, A S SCHULZ, H KESSLER, R LAMMERS, A ULLRICH, C R BARTRAM, J W G JANSSEN: "Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking site" ONCOGENE, vol. 14, no. 22, 5 June 1997, pages 2619-2631, XP002071819 see the whole document	1-4, 6, 7, 10
P, Y	---	5, 11-17, 20-22
A	M SACHS, K M WEIDNER, V BRINKMANN, I WALTHER, A OBERMEIER, A ULLRICH, W BIRCHMEIER: "Motogenic and Morphogenic Activity of Epithelial Receptor Tyrosine Kinases" THE JOURNAL OF CELL BIOLOGY, vol. 133, no. 5, June 1996, pages 1095-1107, XP002071820 see the whole document	1-7, 10-17, 20-22
A	A OBERMEIER, H HALFTER, K-H WIESMÜLLER, G JUNG, J SCHLESSINGER, A ULLRICH: "Tyrosine 785 is a major determinant of trk-substrate interaction" THE EMBO JOURNAL, vol. 12, no. 3, 1993, pages 933-941, XP002071821 cited in the application see the whole document	1-7, 10-17, 20-22

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/06842

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
A	<p>M SPAARGAREN, L H K DEFIZE, J BOONSTRA, S W DE LAAT: "Antibody-induced Dimerization Activates the Epidermal Growth Factor Receptor Tyrosine Kinase"  JOURNAL OF BIOLOGICAL CHEMISTRY,  vol. 266, no. 3, 25 January 1991,  pages 1733-1739, XP002071822  cited in the application  see the whole document  -----</p>	1-22

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/06842

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 27 - 35  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.